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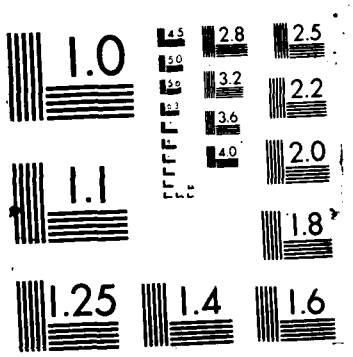
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RIFT VALLEY FEVER VIRUS: MOLECULAR BIOLOGIC STUDIES OF THE M SEGMENT RNA FOR APPLICATION IN DISEASE PREVENTION

ANNUAL REPORT

MARC S. COLLETT, Ph.D.

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The coding capacity and expression strategy of the RVFV M segment were investigated in detail employing cell-free transcription-translation and recombinant vaccinia virus systems. The results indicate that the M segment encodes a primary translation product of 133 kd representing the entire large open reading frame (ORF), which is co-translationally processed. The processed products are the viral glycoproteins G2 and G1, and two newly characterized polypeptides: a glycosylated 78 kd protein, and a non-glycosylated 14 kd protein. The 78 kd protein initiates from the first ATG codon of the ORF and encompasses the entire pre-glycoprotein and glycoprotein G2 coding sequences. The 14 kd protein initiates at the second ATG and represents only pre-glycoprotein sequences. All of these M segment products are largely Golgi-localized in both RVFV-infected and recombinant vaccinia virus-infected cells. Important to expression and subcellular localization of the glycoproteins are the 22 hydrophobic amino acids directly preceding the mature glycoprotein coding sequences. This work demonstrates the utility of recombinant vaccinia viruses in the study of Phlebovirus proteins and gene expression. The recombinant RVFV-vaccinia viruses are also viable live virus vaccine candidates. Work with these viruses has provided further support for the notion that in the RVF disease system, virus neutralization does not correlate with animal protection.

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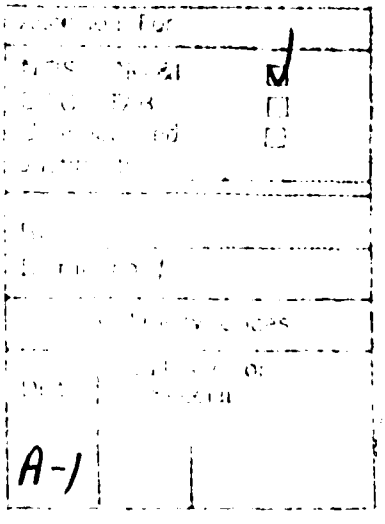
SUMMARY

The purpose of this work is to elucidate the molecular and biological properties of the M segment RNA of RVFV. Work has concentrated on investigations of the coding capacity and expression strategy of the RVFV M segment. We employed cell-free transcription-translation systems and recombinant vaccinia viruses to address these issues. Our results indicate that the M segment encodes a primary translation product of 133 kd (kilodaltons) representing the entire open reading frame (ORF), which is co-translationally processed in virus-infected cells. The processed products are the viral glycoproteins G2 and G1, and two newly characterized polypeptides: a glycosylated 78 kd protein and a non-glycosylated 14 kd protein. The 78 kd protein initiates from the first ATG of the ORF and encompasses the entire pre-glycoprotein and glycoprotein G2 coding sequences. The 14 kd protein initiates at the second in-phase ATG and represents only pre-glycoprotein sequences. All of these M segment products are largely Golgi-localized in both RVFV-infected and recombinant vaccinia virus-infected cells. The 78 kd and 14 kd proteins, and most of the pre-glycoprotein sequence are not required for the proper synthesis, processing, modification, or Golgi localization of the viral glycoproteins. However, the 22 amino acids preceding the glycoprotein coding sequences are important. Deletion of this sequence resulted in abolition of G2 expression, a dramatic decrease in G1 production, and the appearance of G1 at the cell surface. The utility of recombinant vaccinia viruses in the study of Phlebovirus proteins and gene expression is clearly demonstrated. The recombinant RVFV-vaccinia viruses are also viable live virus vaccine candidates. Data indicates that glycoprotein G2 is a critical and sufficient protective immunogen, and further, that virus neutralizing antibody activity does not correlate with protection of animals from disease. Along other lines, work has been initiated toward the development of stable mammalian cell lines capable of producing RVFV M segment polypeptides. Several expression vectors have been constructed and introduced into mammalian cells, and clonal cell lines have been established.



FOREWORD

In performing the recombinant DNA experiments described in this report, the investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

In conducting laboratory animal immunization experiments described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication N. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

A. Introduction. This report summarizes work performed during the second year of this contract. The mission of this contract is to elucidate the molecular and biological properties of the middle (M) genomic segment of Rift Valley fever virus (RVFV), while keeping an eye for the application of the basic research information toward strategies for disease prevention. We had previously cloned and sequenced the entire M segment, and demonstrated that the two viral glycoproteins, G2 and G1, were encoded in this portion of the RVFV genome (1). From this data it was clear that the M segment RNA employed a "negative sense" strategy for gene expression. We went on to characterize the "positive sense" messenger RNA (mRNA) of the M segment during the first year of the current contract. We found the M segment mRNA to lack at its 3' end approximately 112 nucleotides present at the 5' end of the genomic RNA. The 5' end of the mRNA possessed all of the sequences resident at the 3' end of the genomic RNA, but further contained 12-14 nucleotides of presumed cellular origin that extended beyond the end of the genome (2). In other work conducted during this time, we defined and localized four distinct antigenic determinants along glycoprotein G2, three of which were defined by independent virus neutralizing and protective monoclonal antibodies (mAbs) (3). This data, along with the information of the complete nucleotide sequence of the M segment, was used to design and construct bacterial expression plasmids that, when introduced into E. coli, resulted in the production of RVFV glycoprotein analogue polypeptides. These polypeptides could then be evaluated as subunit immunogens. In an alternative approach to RVF vaccine development, we initiated work on the use of live recombinant vaccinia viruses. From initial animal immunization and challenge-protection experiments, the E. coli-produced immunogens elicited only marginal virus neutralization titers, but were capable of providing significant, although not complete, animal protection (4). Mice immunized with a recombinant RVFV M segment-vaccinia virus, on the other hand, developed high virus neutralizing antibodies titers and were completely protected against lethal RVFV challenge (4).

In this second year of investigation, we have further exploited the recombinant vaccinia virus system not only for vaccine candidate development, but also as a research tool to probe aspects of RVFV gene expression. We have de-emphasized E. coli based systems for production of immunogens. Instead, we have incorporated systems for cell-free transcription-translation and mammalian cell expression into our research program to gain further insight into the molecular and biological properties of the RVFV M segment.

B. Coding Capacity and Expression Strategy of the RVFV M Segment. Members of Bunyaviridae appear to employ two fundamentally distinct strategies for gene expression: "negative sense" and "ambisense." The M segment of RVFV appears to employ a negative sense strategy (1,2). However, with respect to the details of RVFV M segment gene expression, several issues arise. The genetic organization of the M segment suggests that the primary translation product of the large open reading frame (ORF) would be a high molecular weight (133 kilodalton; kd) polyprotein possessing the sequences of both glycoprotein G2 and G1. No such precursor protein has ever been detected in RVFV-infected cells. Furthermore, after aligning the amino terminal amino acid sequences of the two viral glycoproteins within this ORF, we previously indicated (1) that two regions might potentially code for additional protein: a region from the first ATG codon of the ORF to the first amino acid of the mature G2 glycoprotein coding sequence, and a second "intragenic" area lying between the coding sequences of the two glycoproteins. The extent to which the former information might be expressed as protein would depend on the selection of one of the five in-phase translation initiation codons that precede the mature glycoprotein coding sequences.

To address the question of the putative "intragenic" region, we attempted to generate antisera in rabbits to synthetic peptides representing the amino acid sequences immediately preceding those of the second (G1) mature glycoprotein sequences. One peptide, representing the amino acids sequence -12 to -1 with respect to the G1 coding sequences (LMLLLIVSYASA), failed to elicit antibodies to itself or any RVFV M segment polypeptide. A second peptide, representing amino acids -27 to -16 upstream of glycoprotein G1 (APIPRHAPIPRY) was immunogenic. Radioimmunoprecipitation analyses demonstrated that antiserum to this peptide recognized glycoprotein G2 and a 78 kd protein described below. Therefore, the extent of any "intragenic" region between the G2 and G1 coding sequences must be minimal, if it exists at all. In fact, the sequences immediately preceding G1 are quite hydrophobic (possibly explaining the insoluble and non-immunogenic nature of the first peptide) and could represent a "leader' or signal sequence of the G1 glycoprotein. Further work will be required to establish this, however.

From the first ATG of the ORF to the beginning of the coding sequences of glycoprotein G2 there exists a potential coding capacity of 17 kd. However, there are four additional in-phase ATG codons between this first translation initiation codon and the glycoprotein gene. To investigate the coding potential of the pre-glycoprotein region and the features of translation initiation of the M segment ORF, we employed two technologies: cell-free transcription-translation and recombinant vaccinia virology.

1. In vitro transcription-translation studies. The in vitro study of the translational features of the M segment mRNA have been hampered by the low abundance and possible instability of this RNA in virus-infected cells. To overcome these difficulties, we have assembled plasmid transcription vectors which incorporate RVFV M segment sequences downstream of the bacteriophage SP6 RNA polymerase promoter. By allowing run-off transcription on the linearized plasmids, abundant RNA may be synthesized in vitro. We have constructed a series of these transcription vectors to explore features of translation initiation and coding capacity of the M segment ORF. This series is diagrammed in Figure 1. Construct 7, 8, 5, and 6 contained M segment sequences beginning just before the first, second, third, and fourth in-phase ATGs, respectively. Construct 9 lacked all pre-glycoprotein sequences, and has had substituted for the first codon of the mature G2 protein (encoding glutamic acid) a methionine (ATG) codon. The RVFV sequences in all transcription vector constructs then extended uninterrupted beyond the authentic termination codon of the M segment ORF to a position approximating that of the 3' end of the authentic mRNA. When subjected to cell-free translation in a reticulocyte lysate, each RNA programmed the synthesis of one principal product, the molecular weight of which approximated that expected for a polypeptide representing the entire ORF present in the construct (between 133 kd for RNA-7 to 120 kd for RNA-9). This suggested that initiation of translation of these transcripts occurred primarily at the first (5'-most) ATG codon and that translation continued to the end of the ORF. Immunologic analyses indicated that both glycoprotein G2 and G1 determinants were present on all of these primary translation products.

Addition of microsomal membranes to these translation reactions resulted in the apparent processing of the primary products. This processing appeared to be co-translational, as addition of membranes after translation failed to yield processed polypeptides. The nature of the processed protein products of these reactions was investigated using several specific immunologic reagents (Table 1). MAb to glycoprotein G1 immunoprecipitated a polypeptide of approximately 68 kd from the processed translation products of all of the RNAs (8, 7, 5, 6, and 9). A G2-specific mAb recognized a processed polypeptide of 63 kd from translation mixtures of RNA-7, -8, -5, and -6, but not RNA-9. In addition, a 78 kd protein and a polypeptide slightly smaller than this was immunoprecipitated by the G2 mAb from RNA-7 and RNA-8 reactions, respectively. A minor G2-specific component of 47 kd was also noted from RNA-7, -8, -5-, and -6.

To determine if processed polypeptides contained amino acids sequences derived from the pre-glycoprotein region of

the M segment ORF, an antiserum (R900; Table 1) generated to a synthetic peptide representing amino acids 40-51 of the ORF was used. R900 serum immunoprecipitated a minor 20 kd protein, a 14 kd protein, and the previously mentioned G2-specific 78 kd protein from RNA-7 reactions. From processed RNA-8 translations, R900 serum recognized the 14 kd protein and the polypeptide slightly smaller than 78 kd. There were no polypeptides immunoprecipitated by R900 from RNA-5, -6, or -9 reactions.

The sum of these cell-free transcription-translation data are schematically presented in Figure 2A. It appeared that polypeptides related and similar in size to glycoproteins G2 and G1 were derived from a precursor polypeptide. In addition to these processed products, additional polypeptides were noted. A 78 kd and a minor 20 kd protein appeared to be initiated from the first ATG of the ORF, while a 14 kd protein appeared as a result of translation from the second in-phase ATG. The unusual translation features of RNA-9 remain to be investigated.

2. Recombinant vaccinia virus studies. A series of recombinant vaccinia viruses possessing M segment sequences similar to those in the above-described transcription vectors was constructed to investigate the coding capacity and expression strategy of the M segment in virus-infected cells (see Fig. 1). In each case, the RVFV M segment sequences were engineered downstream of the vaccinia virus 7.5 K promoter in the background of the WR strain of vaccinia virus.

To identify RVFV-specific polypeptides in cells infected with these recombinant viruses, radiolabeled infected cell lysates were prepared from each and used in standard immunoprecipitation analyses with a panel of immunologic reagents (Table 1). Use of a glycoprotein G1-specific mAb resulted in the immunoprecipitation from all five (7, 8, 5, 6, and 9) recombinant virus-infected cell lysates of a 63 kd/65 kd doublet protein band. This doublet comigrated with the authentic glycoprotein G1 doublet found in RVFV-infected cells. A G2-specific mAb immunoprecipitated a 56 kd protein from cells infected with viruses 7, 8, 5, and 6, but not from virus 9-infected cells. This mAb also reacted with a 78 kd protein found only in virus 7-infected cells. Both the 78 kd and 56 kd comigrated with polypeptides found in RVFV-infected cells, the latter protein being mature glycoprotein G2. In no instance was there observed a sign of any higher molecular weight precursor polypeptide in the recombinant vaccinia virus-infected cells.

To investigate protein expression within the pre-glycoprotein region, immunoprecipitation analyses using R895 and R900 serum were carried out. R895 and R900 represent rabbit antisera generated against synthetic

peptides corresponding to amino acid sequences between the first and second ATG (R895), and between the second and third ATG (R900) (see Table 1). The 78 kd protein, expressed only in recombinant 7-infected cells, was recognized by both R895 and R900, indicating that this protein possessed sequences from between the first and second ATG, and therefore appeared as a result of translation initiation at the first ATG of the ORF. R900 serum, but not R895, immunoprecipitated a 14 kd protein from virus 7 and 8-infected cells. This protein was not found in recombinant 5-infected cells, suggesting that the second ATG of the ORF was employed for the translation initiation of this protein. Immunoprecipitation of RVFV-infected cells with R900 also resulted in the recognition of a 14 kd polypeptide which comigrated with the 14 kd protein found in recombinant 7-infected and 8-infected cells.

These data generated from the study of this series of recombinant vaccinia viruses are summarized in Figure 2B. The results of the cell-free transcription-translation experiments and these vaccinia virus expression studies are in close agreement with the following minor differences. In the cell-free systrem, we found two M segment-encoded polypeptides not seen in RVFV-infected or recombinant virus-infected cells: the high molecular weight precursor polypeptide and a 20 kd protein apparently derived from the pre-glycoprotein region (Fig. 2). The cell-free system allowed us to separate primary translation from the normal co-translational processing of the M segment precursor. The nature and significance of the 20 kd protein remains under investigation.

The 78 kd and 14 kd proteins represent previously uncharacterized gene products of the RVFV M segment. In both cases, potential N-linked glycosylation sites exist in their sequence (Fig. 2). To determine if the 78 kd and 14 kd proteins were glycosylated, two experiments were carried out. Recombinant 7-infected cells were radiolabeled with [^{14}C]-glucosamine, and a cleared lysate immunoprecipitated with either an anti-RVFV antiserum or R900. We found that the 78 kd protein, G2, and G1 incorporated radiolabel, but were unable to detect radiolabeled 14 kd. Alternatively, virus 7-infected cells radiolabeled with [^{35}S]-methionine were used in a similar immunoprecipitation. However, a portion of the immunoprecipitates were treated with endoglycosidases, and then electrophoresed in parallel with untreated samples. We found a change in electrophoretic mobility of the 78 kd protein, G2, and G1 upon endoglycosidase treatment. However, the mobility of the 14 kd remained unchanged. Thus, the 78 kd protein, but not the 14 kd protein, was glycosylated.

As these recombinant vaccinia viruses may have further utility in the future development of vaccines (ref. 4; see

below), it was of interest to compare the relative levels of RVFV glycoprotein production by cells infected with the recombinant vaccinia viruses and authentic RVFV. Using an antigen-capture ELISA assay, we found that compared to authentic RVFV-infected cells, vaccinia virus recombinants 5 and 6 produced about half as much glycoprotein G2 and G1 (normalized for ug total cell protein). Recombinants 7 and 8 yielded only a quarter as much of each glycoprotein. Virus 9-infected cells were severely deficient in glycoprotein G1 production (1%) and, as previously indicated, produced no glycoprotein G2.

The above data demonstrated that recombinant virus 7, which contained the entire ORF of the RVFV M segment, produced the same constellation of M segment-encoded proteins as authentic RVFV (14 kd and 78 kd proteins, as well as the two viral glycoproteins G2 and G1). These proteins appeared to be properly processed and comigrated in SDS-containing polyacrylamide gels with their counterpart polypeptides found in authentic RVFV-infected cells. To determine if the similarity of M segment gene product expression in the recombinant virus-infected cells extended to the subcellular distribution of these proteins, we conducted a series of immunofluorescence localization studies. Using the primary immunologic reagents described in Table 1, we found all four M segment gene products to be almost exclusively Golgi region localized in both RVFV-infected and recombinant 7-infected cells.

Coupled with the protein expression data, these subcellular localization results clearly establish the usefulness of recombinant vaccinia viruses in the study of Phlebovirus-specific polypeptides. Moreover, it indicates that the proteins of the RVFV M segment are themselves Golgi-specific. The L and S genomic segments or their gene products are not required for accumulation of the M segment encoded polypeptides in the Golgi region. The vaccinia virus recombinant data further indicates that RVFV virion maturation is not required for this subcellular distribution of M segment proteins. With respect to this latter point, similar results have been observed in the study of a temperature-sensitive mutant of Uukuniemi virus (5). To further explore the Golgi-specific nature of the M segment proteins, we examined the subcellular localization of these polypeptides in cells infected with each of the recombinant vaccinia viruses possessing progressive 5' deletions of the M segment ORF. Recombinant virus 8 lacks the amino terminal 38 amino acids of the M segment ORF, and expresses in infected cells the 14 kd protein and glycoproteins G2 and G1. Each of these proteins exhibited the same subcellular distribution in recombinant 8-infected cells as seen in RVFV-infected and recombinant 7-infected cells: the 14 kd protein showing Golgi with some cytoplasmic staining and both glycoproteins G2 and G1

revealing exclusive Golgi complex localization. The M segment coding sequences in recombinant virus 5 begin at the third in-phase initiation codon and consequently fail to produce both the 78 kd and 14 kd proteins. Glycoproteins G2 and G1 produced in virus 5-infected cells remained Golgi complex localized. Vaccinia recombinant 6 initiates RVFV M segment expression at the fourth in-phase ATG, 22 codons upstream of the mature glycoprotein coding sequences. Both glycoprotein G2 and G1 were localized to the Golgi complex.

In recombinant virus 9, the entire pre-glycoprotein region, including the 22 amino acid hydrophobic sequence preceding glycoprotein G2, has been removed. Furthermore, the first amino acid of glycoprotein G2 (glutamic acid) was replaced with methionine. This recombinant failed to produce the 78 kd, 14 kd, and G2 proteins based on radioimmunoprecipitation analyses. These results were confirmed by immunofluorescence studies. Glycoprotein G1, expressed at a reduced level, was not detected in the Golgi region, but rather appeared on the cell surface. In contrast, neither glycoprotein G1 nor G2 was detectable on the cell surface in cells infected with recombinant viruses 7, 8, 5, or 6. The novel localization of glycoprotein G1 on the surface of recombinant virus 9-infected cells did not appear to be due to any gross changes in protein size or glycosylation, as the immunoprecipitated G1 doublet protein was indistinguishable from that produced in authentic RVFV-infected cells. Thus sequences upstream of the mature glycoprotein G1 must dictate the Golgi localization of this protein.

The immunofluorescence data indicated that, with the possible exception of some of the 14 kd protein, all of the RVFV M segment encoded polypeptides were closely associated with membraneous organelles of the cell. As an initial step in understanding the nature of these associations, we employed the Triton X-114 partitioning procedure of Bordier (6), in which integral membrane proteins are exclusively extracted into a detergent phase while all other proteins appear in the aqueous phase. Using this technique with authentic RVFV-infected cells, the 78 kd and 14 kd proteins and glycoprotein G2 appeared to partition into both the aqueous and detergent phases. Thus, there existed populations of these proteins that were integrally associated with Golgi membranes and that were more loosely Golgi-associated. However, in all experiments, we found glycoprotein G1 to partition completely in the detergent phase, indicating its exclusive integral membrane character, as well as internally verifying the fractionation procedures. Similar Trition X-114 partitioning results were obtained for the Golgi-localized RVFV M segment proteins expressed in vaccinia recombinant virus 7-infected cells. The plasma membrane association

of glycoprotein G1 in recombinant virus 9-infected cells was analyzed by Trition X-114 partitioning. As with Golgi complex-associated glycoprotein G1 found in RVFV-infected and recombinant vaccinia virus-infected cells, cell surface-associated glycoprotein G1 partitioned completely as an integral membrane protein. Table 2 provides a summary of data discussed to this point regarding the M segment recombinant vaccinia viruses.

C. Strategies for Protection against RVFV Infection.

1. Bacterially-produced-RVFV-subunit-immunogens. The descriptions of *E. coli* expression plasmids possessing numerous variations of RVFV M segment sequences have been provided before (4; see First Annual Report). Evaluation of sera from mice immunized with several of the *E. coli*-produced glycoprotein analogues, formulated in various manners, resulted in plaque reduction neutralization titers (80% PRNT) that were in all cases marginal, with the highest values reaching only 40. In view of these results and other data, we have chosen to de-emphasize work in this area so that our resources may be directed toward more promising areas.

2. Mammalian cell production of RVFV subunit immunogens. One potentially promising area we have explored over the past year has been the construction of expression vectors suitable for stable maintanence in mammalian cells. The establishment of stable mammalian cell lines expressing RVFV M segment polypeptides has several attractive features. First, RVFV proteins produced in mammalian cells should be correctly processed, modified, and folded into their "native" structure. Production of such "natural" polypeptides in the absence of virus infection (by either RVFV or recombinant vaccinia viruses) has certain safety, as well as subunit protein production advantages. Finally, such cell lines may also be useful for basic biologic and immunologic research issues.

To date, much of our effort in this area has consisted of obtaining a set of the fundamental genetic elements required for mammalian cell expression vectors. The elements are listed in Figure 3, and are all generally cassettable. Using these elements and the cloned RVFV M segment, we have constructed several expression plasmids. These have been transfected into several cell types, and using the appropriate dominant selection procedures, we have isolated clonal cell lines. Initially, we attempted to insert sequences encompassing the entire ORF of the M segment downstream of each of the three promoter-enhancer elements indicated in Figure 3. To date we have been unsuccessful in our attempts to establish continuous mammalian cell lines capable of expressing protein from the entire M segment. Analysis of the M segment sequence for concensus RNA splicing sites revealed three potential

splice donor sites (87% homology with concensus) and numerous potential splice acceptor sites. We currently have no direct data as to their actual use. We have, however, constructed a series of mammalian cell expression vectors in which only portions of the M segment are present, attempting to omit putative splice donor sequences. COS cells have been transfected with a vector containing a truncated G2 gene (lacking 70 carboxy-terminal amino acids) under the control of the SV40 early promoter and an SV40-neo transcription unit for dominant selection in G418. Immunofluorescence analyses of numerous cloned cell lines indicated that from 5% to 50% of the cells in a given population were positive for the G2 protein, which in all cases was Golgi localized. Of those cells that were positive, many were present in multinuclear cell clusters. A G2 protein of the predicted size was readily immunoprecipitated from cell lysates of these cultures. However, as these clonal lines were followed over time, the percentage of positive cells appeared to decrease. We currently feel expression of this G2 protein analogue is toxic (fusogenic?) to cells. A vector is currently under construction in which expression of this truncated G2 gene is under the control of the inducible MMTV LTR promoter. Alternatively, we have established CHO cell lines containing the same SV40-truncated G2 expression unit, but employing an SV40 driven dhfr transcription unit for dominant selection in methotrexate (MTX). Clonal CHO cell lines appear to be healthier than the above described COS cell lines and exhibit a higher proportion of cells positive for Golgi-localized, G2-specific immunofluorescence, although the intensity of the fluorescence was weaker. With these CHO cell lines, the G2 analogue protein was also readily identified by immunoprecipitation.

3. Live recombinant RVFV-vaccinia viruses. We have previously demonstrated the potential utility of one recombinant RVFV-vaccinia virus (vGSRV-5) as a live virus vaccine candidate for protection against RVF (4). Since that time we have constructed additional recombinant viruses, each expressing a slightly different portion of the M segment ORF and polypeptides encoded therein. In a mouse challenge-protection experiment conducted at the Virology Division, USAMRIID by J.M. Dalrymple and co-workers, these new recombinant RVFV-vaccinia viruses were evaluated. Mice received a single inoculation of vaccinia virus by tail scarification, and 28 day post-vaccination serum was assayed for 80% PRN titers. From individual mice in each immunization group, titers ranged from <10 to 10,240 (Table 3). With one exception, all the vaccinia virus recombinants were able to completely protect mice against a lethal RVFV challenge. This included the virus vGSRV-G2,

which expressed only the G2 glycoprotein, confirming previous results indicating G2 may be a sufficient protective immunogen. The exception to complete mouse protection was vSCRV-9. This virus fails to express G2 and expresses only low levels of glycoprotein G1. Thus, vSCRV-9 may not be protective due to its low level immunogen expression. Alternatively, G1 may not be involved in protective immunity. Noteworthy here may be the fact that in vSCRV-9-infected cells, the G1 protein is expressed on the cell surface. With all other recombinant viruses and authentic RVFV, glycoproteins were localized exclusively in the Golgi region of the infeceted cells (Table 2). As all of the animals immunized with vSCRV-9 exhibited significant neutralizing titers, there appears to be no correlation between neutralization and protection in this system.

4. Antisense RNA cells. To determine if RVFV infection of cultured cells can be inhibited by mechanisms involving "antisense" RNA, we have constructed a series of CHO cell lines. Plasmid vectors have been assembled in which the RSV LTR promoter-enhancer directs the transcription of a single RNA composed of either: (i) the dhfr gene coding sequences (dhfr), (ii) the dhfr sequences followed behind the translation termination codon of dhfr by sequences representing the first 302 nucleotides complementary to the 3' end of the RVFV M genomic RNA (dhfr-A), or (iii) dhfr followed by 302 nucleotides representing sequence complementary to the 5' end region of the M segment mRNA (dhfr-B) (see Fig. 4). The idea is that cells producing the dhfr-A chimeric RNA, selected on the basis of the dhfr-positive phenotype, might be resistant to virus infections due to the antisense RNA interferring with viral transcription and/or replication (by complexing with viral-sense RNA). Similarly, dhfr-B RNA-producing cells may prevent RVFV mRNA translation, thereby inhibiting infection. The use of dhfr as the selectable marker, to which the antisense sequences of directly fused, will allow gene amplification of both sequences upon methrotrexate (MTX) selection, thereby allowing us to regulate the levels of antisense RNA in the cells.

CHO cell lines have been established that possess these plasmid vectors. These cell lines are being cultured in increasing concentrations of MTX so as to amplify the level of dhfr-containing RNA transcripts. RNA from cells maintained in 3 μM MTX were screened by Northern format hybridization for expression of RVFV sequences and were all found to be positive; however, the relative levels of transcripts in the dhfr-A cells (RVFV mRNA-sense transcripts) were significantly lower than those in the dhfr-B cells (RVFV vRNA-sense). In an initial attempt to determine if RVFV glycoprotein expression might be

inhibited in these antisense cell lines, we infected these cells with RVFV-vaccinia recombinant virus vSCRV-7. We saw no difference in the level of synthesis of RVFV M segment polypeptides among the control (dhfr only), dhfr-A, and dhfr-B cells.

D. Research Directions for the Upcoming Year. Considerable effort has been put forth this past year in elucidating the coding capacity and expression strategy of the RVFV M segment. To address these basic questions we employed cell-free transcription-translation systems and recombinant vaccinia viruses. Our results to date suggest that the M segment encodes a primary translation product of approximately 133 kd representing the entire large open reading frame (ORF), which is co-translationally processed. Four processed polypeptides have been identified: 78 kd, 14 kd, glycoprotein G2, and glycoprotein G1. It appears that the 78 kd protein initiates from the first ATG codon of the ORF and encompasses the entire pre-glycoprotein and glycoprotein G2 coding sequences. The 14 kd protein initiates at the second ATG codon in the ORF and represents only the pre-glycoprotein sequences. There appears to be no significant coding information between the carboxy terminus of G2 and the amino terminus of G1. Thus, we have established the coding capacity of the M segment RNA. However, several questions remain regarding the expression strategy of this RNA and the functional roles of its gene products. The significance using alternative translation initiation codons to encode different polypeptides from the same ORF remains unclear. To further address these issues, we have initiated work on the construction of oligonucleotide-directed mutant plasmids. Specifically, our plan is to change select MET (initiation codon) residues to LEU or ILE, reconstruct transcription and vaccinia transfer vectors to contain the entire M segment with the desired mutation, and then analyze the features of polypeptide expression and processing in the respective systems. The role of the two newly characterized M segment proteins, 78 kd and 14 kd, as well as the pre-glycoprotein sequences, remains to be established. The 78 kd and 14 kd proteins are not required for glycoprotein synthesis, processing, modification, or Golgi localization. We plan to explore the immunologic features of these two proteins, independent of the viral glycoproteins in an effort to gain clues as to their role in RVFV infection.

Removal of pre-glycoprotein sequences up to 22 amino acids prior to mature glycoprotein G2 similarly had no effect on the viral glycoproteins. However, removal of the 22 amino acid hydrophobic sequence directly preceding G2 abolished G2 production and resulted in G1 expression at the cell surface. Obviously, these sequences are important for RVFV glycoprotein expression and subcellular distribution. We plan to pursue the role of these seuqences as well as sequences resident in G2, with the notion that there may exist Golgi-specific retention

signals in the M segment sequence. Not only will these investigations be of inherent interest from the cell biology point of view, they may also have important implications for the design of new recombinant vaccinia virus vaccine candidates. The release from the Golgi and expression at the cell surface of the critical immunogen glycoprotein G2 may have profound effects on the efficacy of the product.

Similarly, such work will impact our efforts in establishing stable mammalian cell lines that produce useful RVFV subunit immunogens. In addition, careful consideration for potential RNA splicing signals and regulated gene expression will be included in our work in this area.

With the current series of recombinant vaccinia viruses and the impending availability of mammalian cells expressing RVFV M segment gene products, we plan to begin exploring the cellular immune responses in animals to these polypeptides.

RVFV antisense RNA from the various antisense CHO cell lines will be quantified, and attempts will be made to determine if the RNA from these cells is capable of inhibiting the cell-free translation of an appropriate RVFV M segment RNA transcript in vitro. Additional (new) antisense expression constructs will also be considered. The characteristics of authentic RVFV infection, replication, and protein synthesis in these cells will be analyzed.

We are quite satisfied with and extremely excited about our progress, experimental results, and research directions over the past year. We look forward to advancing our ongoing work and to addressing the many issues brought on by our past discoveries in the forthcoming year.

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TABLE 1

Immunologic reagents used to study RVFV M segment proteins

Name	Description[1]	Protein Specificity
R895	Rabbit serum against pre-glycoprotein synthetic peptide 16-30	78 kd
R900	Rabbit serum against pre-glycoprotein synthetic peptide 40-51	78 kd 14 kd
G2	MAb 4-39-CC, recognizes G2 sequence 127-146 (ref. 3).	78 kd G2(56 kd)
G1	MAb 4G11, recognizes unmapped epitope of G1	G1(63/65 kd)

[1]Numbers represent amino acid coordinates of RVFV M segment ORF (see ref. 1).

TABLE 2

Summary of characteristics of recombinant RVFV-vaccinia virus

virus	Proteins Produced	Expression[1] Level	Subcell[2] local	Integral[3] memb
RVFV	78 kd	ND	Golgi	+/-
	14 kd	ND	Golgi/cyto	+/-
vSCRV-7	G2	0.24	Golgi	+/-
	G1	0.24	Golgi	+
	14 kd	ND	Golgi/cyto	+/-
vSCRV-8	G2	0.28	Golgi	+/-
	G1	0.22	Golgi	+
vGSRV-5	G2	0.49/0.51	Golgi	+/-
vSCRV-6	G1	0.49/0.44	Golgi	+
vSCRV-9	G1	0.01	cell surface	+

[1]Relative glycoprotein expression levels were calculated from antigen-capture ELISA data, with the levels in RVFV-infected cells arbitrarily assigned a value of 1. ND = not determined.

[2]Subcellular localization of the individual polypeptides was determined by immunofluorescence analysis using the primary reagents presented in Table 1. cyto = cytoplasm.

[3]The integral membrane nature of the proteins was determined by partitioning in Triton X-114 (6). +/- indicates both integral and non-intregal protein distribution; + indicates exclusive integral membrane partitioning.

TABLE 3

Immunization of mice with recombinant RVFV-vaccinia viruses

Immunizing[1] virus	Proteins Produced	PRNT[2] Ave.	PRNT[2] Range	Protection Survivors/Total	Protection %
vSCRV-7	78 kd/14 kd/G2/G1	1400	<10-5120	25/25	100
vSCRV-8	14 kd/G2/G1	2500	<10-5120	24/24	100
vGSRV-5	G2/G1	3100	<10-5120	25/25	100
vSCRV-6	G2/G1	3000	<10-5120	25/25	100
vSCRV-9	G1	2200	160-5120	4/25	16
vGSRV-G2[3]	G2	3200	10-10,240	24/24	100
control[4]	---	----	---	1/15	7

[1]On day 0, animals received 2 x 10^6 PFU of the indicated recombinant vaccinia virus by tail scarification. Animals were bled on day 28, challenged by intraperitoneal injection of 10-50 LD_{50} of RVFV (ZH501), and observed for 21 days following challenge.

[2]Plaque reduction neutralization titers (PRNT) determined on the 28 day bleeds are expressed as the highest dilution of serum inhibiting greater than 80% of the virus plaques present in control cultures. The average titer and range of titers is presented.

[3]Recombinant vGSRV-G2, not described in the text, possesses RVFV M segment sequences from the third in-phase ATG to the end of the G2 protein coding sequences and expresses properly processed and modified glycoprotein G2.

[4]Control, unimmunized mice, challenged with RVFV as above.

FIGURE 1

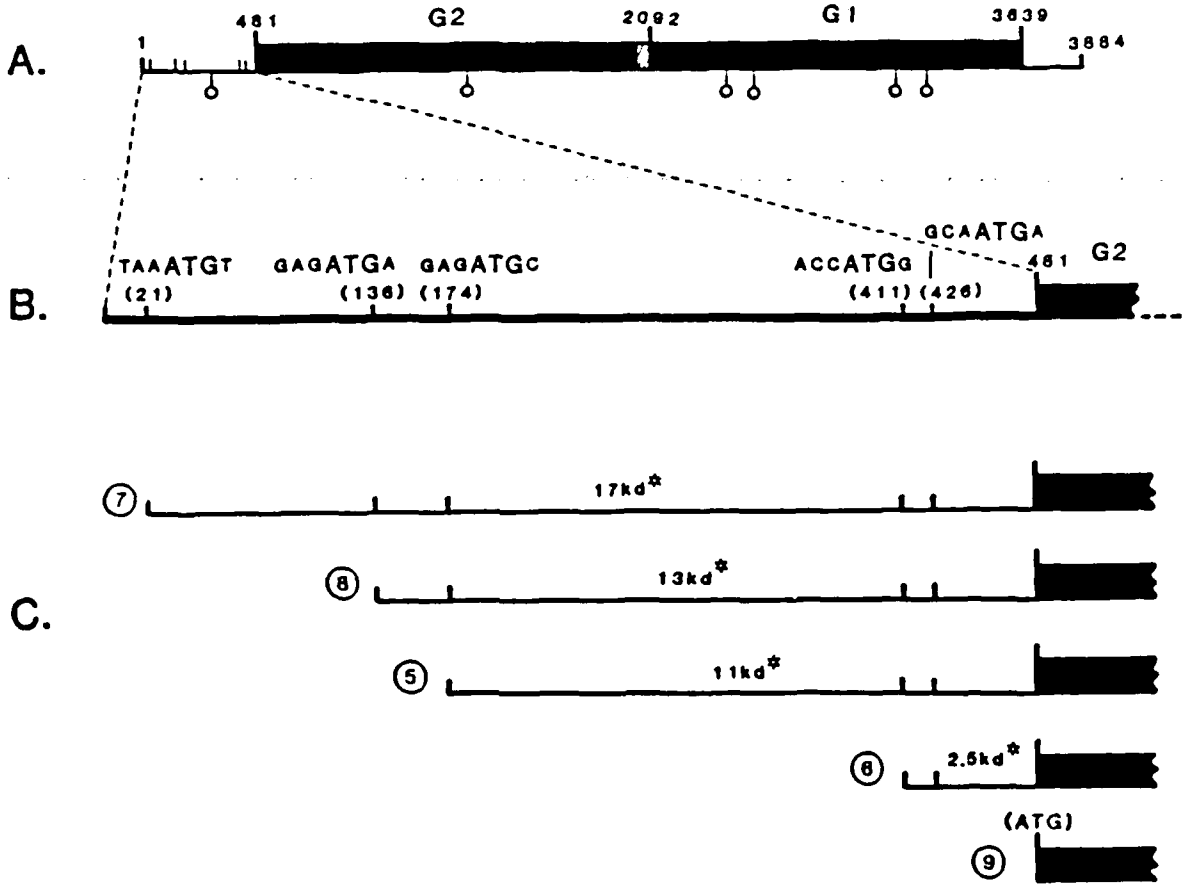


Figure 1. RVFV M segment sequences present in transcription vectors and recombinant vaccinia viruses. A. Molecular organization of the RVFV M segment RNA. Numbers represent nucleotide coordinates of the M segment for the start of the mature glycoprotein coding sequences (481 and 2092 for G2 and G1, respectively) and the termination codon of the large ORF (3639). The solid bars indicate the glycoprotein coding regions. The five in-phase ATGs preceding the start of the mature glycoprotein sequences are denoted with vertical tic marks. Potential N-linked glycosylation sites are also shown (♁). B. Expanded representation of the pre-glycoprotein region showing the position and context of the five potential initiation codons. C. Schematic representation of the 5' M segment sequences present in the transcription vectors and recombinant vaccinia viruses. The protein coding potential (in kilodaltons, kd) from the first available ATG to the beginning of the G2 coding sequences for each construct is indicated (*).

FIGURE 2

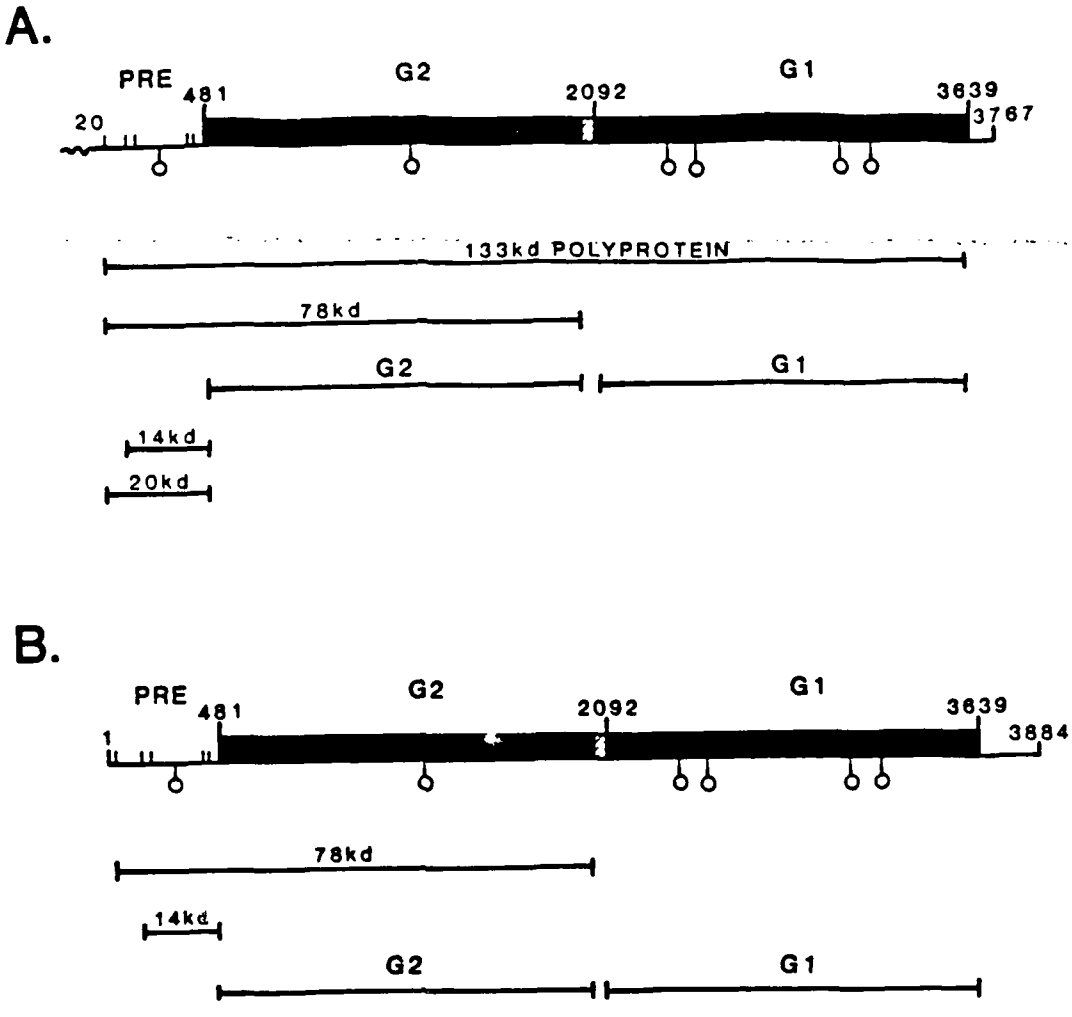
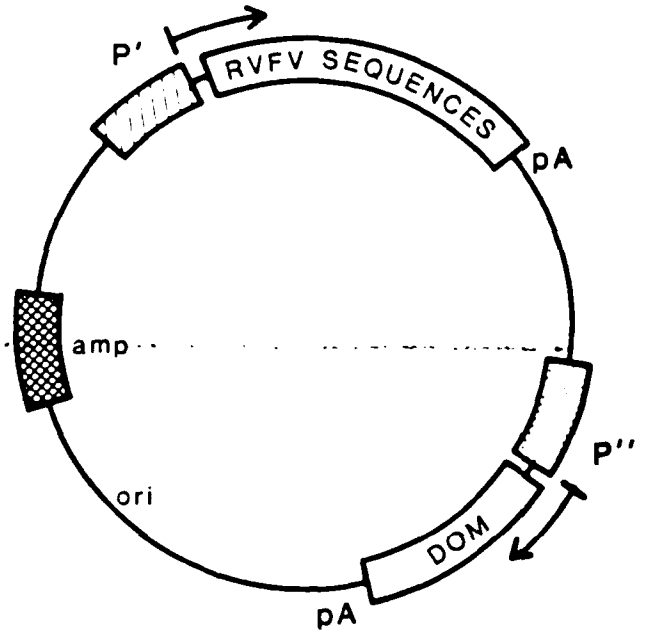


Figure 2. Schematic representation of the RVFV M segment RNA summarizing the polypeptides observed in cell-free translation reactions (A) and in recombinant vaccinia virus-infected cells (B). The numbers and designations are as in Figure 1. The coding regions encompassed by the respective proteins are indicated by the thin lines. All carboxy-terminal positions are estimations.

FIGURE 3

Generalized structure of mammalian cell vectors for expression of RVFV M segment polypeptides



P': promoter-enhancer element for RVFV sequences: SV40 early
RSV LTR
MMTV LTR

pA: poly A addition signal

P": promoter-enhancer element for dominant selectable marker:
SV40 early
RSV LTR

DOM: dominant selectable marker gene:
gpt(HAT resistance)
neo(G-418 resistance)
dhfr(MTX resistance)

amp: ampicillin resistance gene for selection in E. coli

ori: Col E1 replicon for replication in E. coli

FIGURE 4

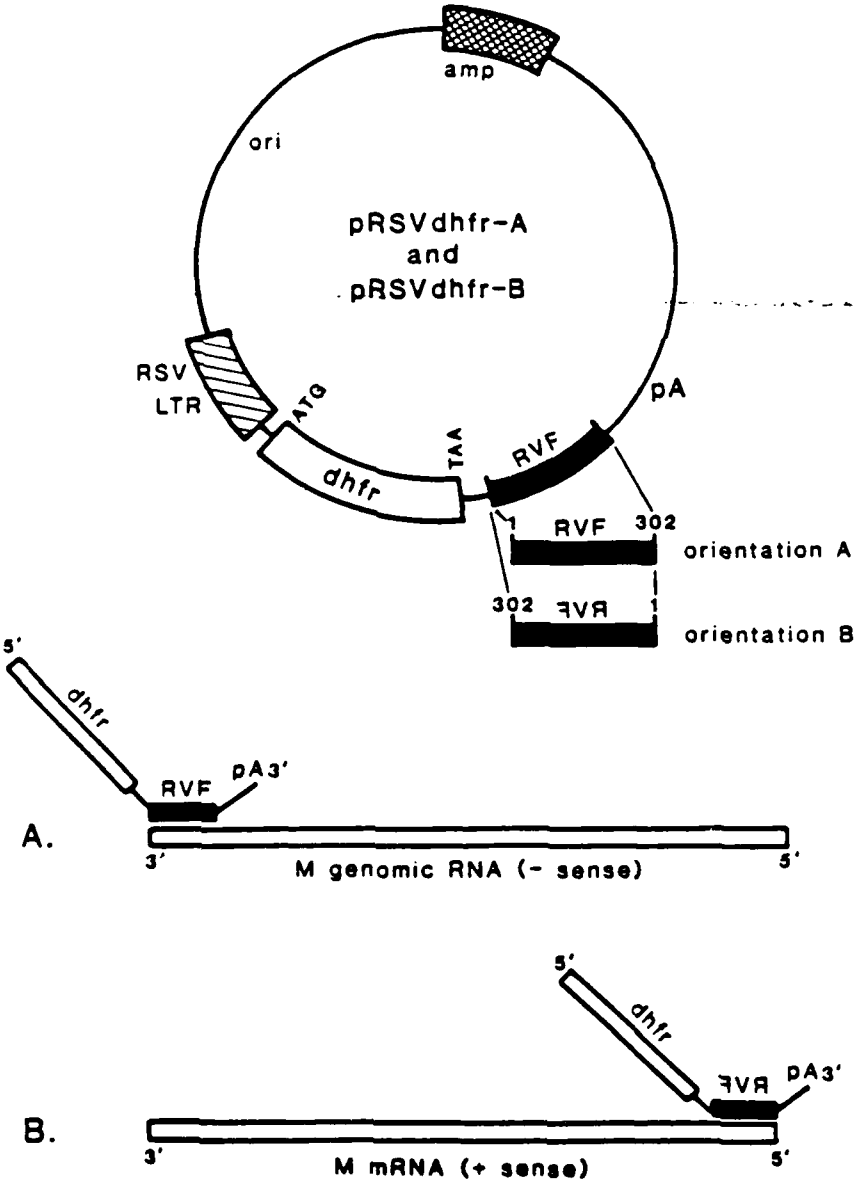


Figure 4. RVFV antisense RNA vectors. Three mammalian cell plasmid vectors were constructed. One contains the RSV LTR promoter-enhancer directing the synthesis of the dihydrofolate reductase (dhfr) gene. In the two other vectors, RVFV sequences from nucleotide 1 to nucleotide 302 have been inserted in either *orientation downstream of the termination codon* (TAA) of dhfr and upstream of the poly A addition site (pA). The resultant chimeric RNAs produced by the later two transcription units could interact with the viral M genomic RNA (A) or the M segment mRNA (B) as indicated.

END

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